# MONITORING OF ANTIBODIES AND ANTIGENS TO AFRICAN SWINE FEVER VIRUS (ASFV) IN PIGS FROM KADUNA AND PLATEAU STATES

BY

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## DECLARATION

I hereby declare that the work in this thesis is original. It was carried out by me under the supervision of Prof. J. U. Umoh (Department of Veterinary Public Health and Preventive Medicine), Prof. J. Adekeye (Department of Veterinary Pathology and Microbiology) and Dr. K. A. Majiyagbe (Virology Division, National Veterinary Research Institute, Vom). Reference is made to work of other investigators and were duly acknowledged. No part of this thesis has been submitted elsewhere for a degree or diploma.



**Luther Nanfwang Joram**

## CERTIFICATION

This thesis entitled "Monitoring of Antibodies and Antigens to African Swine Fever Virus (ASF) in Pigs from Kaduna and Plateau State" by Luther Nanfwang Joram, meets the regulation governing the award of Master of Science Degree of Ahmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

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## DEDICATION

To the memory of my late father, Mr. Luther Sale Magaji who died during the course of this present project work. May his soul rest in perfect peace with the Lord Jesus until we meet to part no more, Amen.

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## TABLE OF CONTENT

Title Page ..... i
Declaration ..... ii
Certification ... ..... iii
Dedication ..... iv
Acknowledgment ..... v
Table of Content ..... vii
List of Tables ..... ix
List of Figures ..... x
Abstract ..... xi
CHAPTER ONE
1.0 Introduction ..... 1
CHAPTER TWO
Literature Review ..... 3
2.1 Historical Background ..... 3
2.2 Epizootiology ..... 5
2.3 Virus Properties ..... 7
2.4 Structure of the Viral Particle ..... 8
2.5 Structural Proteins ..... 9
2.6 Enzyme Activities ..... 11
2.7 Nucleic Acid Metabolism ..... 12
2.8 Immune Response to ASFV by Host ..... 13
2.9 Immune Response Modulation ..... 17
2.10 Antigenic and Immunological variation in ASF Viruses. ..... 17
2.11 ASFV Taxonomic Position ..... 19
2.12 Clinical Disease and Pathology ..... 21
2.13 Diagnosis ..... 24
2.14 ASF Virus Detection ..... 24

2.15 ASF-DNA Detection ..... 25
2.16 Antibody Detection ..... 26
2.17 Treatment ..... 27
2.18 Prevention ..... 28
2.19 Update of the Situation of ASF in Nigeria ..... 29

CHAPTER THREE

MATERIALS AND METHODS

3.0 Pigs: ..... 30
3.2. Sample Collection: ..... 30
3.3 Methods ..... 31
3.3.1 Indirect Enzyme Linked Immunosorbent Assay (I-ELISA) ..... 31
3.3.2. Direct Immunofluorescence ..... 34
3.3.3 DNA Extraction and Polymerase Chain reaction (PCR). ..... 35

CHAPTER FOUR

4.1 Incidence of Antibodies to ASFV in pigs ..... 40

CHAPTER FIVE

Discussion, Conclusions and Recommendations ..... 46
5.1 Discussion ..... 46
5.2 Conclusion and Recommendation ..... 48
References ..... 50
Appendix ..... 62

## LIST OF TABLES

TABLE: 4.1. Frequency of Antibodies to ASFV in pigs sera from Kaduna and Plateau States. ..... 42

TABLE. 4.2 Sex Distribution of Antibodies to ASFV in pigs from Plateau and Kaduna States. ..... 43

TABLE. 4.3 Results of antigens to ASFV in swine tissue samples tested from some parts of Kaduna and Plateau state ..... 44

## LIST OF FIGURES

Figure 1: PCR Analysis of DNA extracted from pig tissue suspected of ASF ..... 45

## ABSTRACT

The prevalence rate of antibodies to African swine fever virus (ASFV) in parts of Plateau and Kaduna states was investigated by indirect enzyme linked immunosorbent assay (I-ELISA). Of a total of 590 pig sera tested from Plateau state, 15 (2.5%) tested positive to ASFV antibodies. ASFV antibodies were detected in serum samples from 3 out of the 4 local government areas of the southern part of Kaduna state with Kaduna south local government area having the highest rate of 29.4% and Jama'a having the lowest rate of 6%. None or the serum samples from Kachia local government area had antibodies to ASFV. The sex specific rate for male (7.4%) significantly differs from that of the female (3.6%). ($X^2$ = 5.721, P<0.05). The occurrence of antigens to ASFV in the study area was determined by direct immunofluorescence (DIF). Nine out of 20 tissue samples tested from Plateau state were positive for ASFV antigens while 5 out of 6 tissue samples tested from Kaduna state were positive. Those tissues that tested negative for ASFV antigens by DIF were retested for virus deoxyribonucleic acid (DNA) by polymerase chain reaction (PCR). The results showed that the 9 out of the 10 tissues tested were positive for virus DNA genome except one tissue (i.e. 1 spleen). This study indicates the presence of African swine fever (ASF) in Plateau and Kaduna states, with the conclusion that ASF may be a major cause of mortality limiting pig production.

## CHAPTER ONE

### 1.0 INTRODUCTION

African swine fever (ASF) is a viral disease of domestic pigs. Recently, it has been described as one of the most important problems of the pig industry in Nigeria (Majiyagbe, 1999). Its manifestation is like a haemorrhagic fever with very high mortality, usually close to 100% especially with the African isolates (Montgomery, 1921; De Tray, 1963). The course of the disease is predominantly peracute or acute. The acute form of the disease was the one observed when ASF was first seen in Spain and Portugal (Polo Jover & SanChez Botija, 1961). Affected pigs show a temperature of over 40°C for 1 or 2 days whilst behaving normally; then they gradually become anorexic and depressed. At times the fever may rise to 42°C and persists until death, although temperatures may sometimes become subnormal in moribund animals (Mebus, 1988).

The disease was first described in Kenya (Montgomery, 1921) and since then has been reported in a large number of African countries (Wilkinson, 1981; Plowright, 1984). Pigs of all ages and breeds are the only domestic animals that are naturally infected by the virus with equal susceptibility. However, European wild boars (*Sus scrofa*) have been found dead with natural disease in Spain and Portugal (SanChez-Botija, 1982) and Sardinia (Contini *et al*, 1982) and feral pigs in Florida are also susceptible to infection (McVicar *et al*, 1981).

ASF is caused by a large unclassified DNA virus that has no relation with any other viral pathogens of domestic animals. Although ASFV was once

tentatively classified as an Iridovirus (Vinuela, 1985), it is currently considered to be the only known member of an as yet unnamed separate genus - "African swine fever - like viruses" in the family poxviridae (Murphy *et al*, 1995). Infection with the virus invokes an immune response with the production of antibodies but there is no evidence that these antibodies are capable of neutralizing virus infectivity (De Boer, 1967; De Boer *et al*, 1969).

Therefore, the eradication of ASF requires rapid and specific diagnosis since an effective vaccine does not exist despite several years of research efforts. It was against this background that the idea of this present pilot study was conceived with the under listed objectives:

i. To investigate and document the occurrence of antibodies to ASFV in pigs from parts of Plateau and Kaduna states.

ii. To detect ASFV antigens from tissues.

iii. To detect virus genome from tissues using polymerase chain reaction (PCR).

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Historical Background

African swine fever (ASF) was first described as an entity separate from classical swine fever in Kenya by Montgomery in 1921. Shortly afterwards, the disease was described from Angola and Congo (Manso-Ribeiro *et al*, 1958). In Angola, it was remarked that the disease afflicted village pig and that certain indigenous breeds of pigs appeared to survive in greater numbers than expected (Anon, 1998). Since then there has been reported outbreaks in a large number of African countries (Wilkinson, 1981; Plowright, 1984). With the exception of Lesotho and Swaziland, where the disease has never been observed, all countries in the regions of Southern, Eastern, and Central Africa are affected (Anon, 1998). In some of these countries, African swine fever virus (ASFV) is present in certain areas only in the reservoir hosts, the warthog, and the soft tick, and the pattern of the disease is sporadic. In 1996, Coted'Ivoire suffered an epidemic of ASF and in 1997 Benin, Togo and Nigeria joined the ranks of the infected countries in 1998 (Anon, 1998).

In 1957 and again in 1959, ASF was exported from Africa to Europe, and subsequently to Caribbean Islands and Brazil via infected pork products (Anon, 1998). The devastating effect of the disease on countries with well developed commercial pig industries highlighted its seriousness and importance and resulted in intensive research on the virus that continues till today (Anon, 1998).

The first real breakthrough in ASF research after Montgomery was made by W. A. Malmquist at the end of the 1950's (Malmquist and Hay, 1960). Working in Kenya he discovered the haemadsorption and cytopathic effects in buffy coat and bone marrow culture which, were the foundation on which virtually all laboratory work has been based. Diagnostic techniques, serology, studies on pathogenesis, immunology, epidemiology and molecular biology were all suddenly made technically and economically feasible.

In the absence of a vaccine, control depends on early observation and rapid diagnosis, followed by a stamping-out policy based on the slaughter of all infected and in-contact pigs. In addition, strict quarantine and control of movement of pigs and products are required. Unfortunately, this policy has proved very difficult if not impossible to apply in most of the countries affected. The reason vary from country to country, but always include financial limitations (mainly inability to compensate owners adequately in order to engage their co-operation). These reasons make outbreaks usually very expensive to control. The outbreak in Malta which cost about $29 million for both eradication and restocking only affected of about 70,000 pigs (Wilkinson *et al,* 1980). Also in Belgium, the number of pigs killed and destroyed was 34,895, and the cost of all the operations connected with eradication was $4.7 million (Yubero and Vizcaino, 1988).

## 2.2 Epizootiology

ASF virus is maintained in Africa by a sylvatic cycle involving the natural host warthog (*Phacochoerus aethiopicus)*, bush pigs (*Potamochoerus porcus*) and soft, or eyeless tick vector of the *Ornithodoros moubata* complex that live in the burrow of warthog (De-Tray, 1963). A single isolation from a giant forest hog (*Hylochoerus meinertzhageni*) has also been recorded (Heuschele and Coggins, 1965). Although the report by Cox (1963) of the isolation of ASF virus from hippopotamus, porcupine and hyaena has not been confirmed (Stone and Heuschele, 1965), the existence of alternative hosts could explain the peculiarities of the epizootiology of ASF in some areas (Plowright, 1977; Thomson *et al,* 1981). High infection rates occur in most warthog populations although some groups are free from the infection (Thomson *et al,* 1980). The distribution of the tick vector determine the distribution of the virus (Thomson *et al,* 1980). Within tick populations, infection rates increase with size and age and adult females have higher infection rates than adult males (Plowright, 1977; Thomson *et al,* 1981). Upon infection of the vector, the virus replicates initially in the gut and then spreads to the salivary glands and reproductive organs (Greig, 1972); as a result, excretion occurs through salivary and coital fluids. Transovarian and sexual transmission of the virus in the vector has been described (Plowright *et al,* 1970). Since the virus is not maintained in the warthog population by either vertical or horizontal transmission (Plowright,

1977), infection of warthogs results from bite from infected ticks (Thomson *et al*, 1980).

The most likely source of infection to domestic pigs in Africa is the tick. Plowright (1977) has reported that some ticks may be infected persistently for up to 3 years and may transmit the infection to pigs during this time. Infection of pigs after eating tissues of acutely infected warthogs had been reported (Thomson *et al*, 1980). Movement of infected pigs is another important risk factor in the spread of ASF, since the pigs are highly infectious for several days before the onset of clinical signs as well as during the course of the acute disease. Levels of the virus in the tissue are high (Plowright *et al*, 1968) and because of its great stability in blood and excretions, contamination of waste food, people, equipment, vehicle, etc. may be sufficient for spread to occur. Early efforts to demonstrate airborne transmission were unsuccessful (Montgomery, 1921) but recent studies have shown that airborne transmission can occur over short distances (Wilkinson *et al*, 1977). Recovered pigs are resistant to challenge with homologous virus (Ruiz-Gonzalvo *et al*, 1986), but challenge with related heterologous viruses may result in virus replication without clinical signs or virus reactivation may occur if pigs are subjected to stress and the levels of virus produced may be sufficient for transmission to occur by the shedding of blood, contamination of waste food or infection of ticks, although transmission by recovered pigs not showing clinical signs is rare (Sanchez-Botija, 1982). The spread of ASF from Africa to other continents has occurred

mainly as a consequence of feeding pigs with infected pig meat or meat products, introduced through ports or airports or by travellers bringing infected meat products from ASF endemic areas (Wilkinson *et al*, 1980). In this way, ASF has spread to the Western Hemisphere, from which it has apparently been eradicated and to Southern Europe where until recently, it has become established as an endemic disease in the Iberian Peninsula and Sardinia.

There is no difference in the distribution of infection amongst pigs in terms of age or sex. Pigs of all ages and sexes are equally susceptible (Wilkinson, 1981; Plowright, 1984). It has also been reported (Anon, 1998) that certain indigenous breeds of pigs appeared to survive in greater numbers than exotic breeds.

## 2.3 Virus Properties

African swine fever virus (ASFV) is a cytoplasmic icosahedral deoxyribo-virus, tentatively classified as an iridovirus (Vinuela., 1985) that infect porcine species. ASF virus replicates primarily in mononuclear phagocyte system cells (Maimquist & Hay, 1960; Minguez *et al*, 1988). ASF virus replication has also been described in endothelial cells (Wilkinson & Wardley, 1978), hepatocytes (Sierra *et al*, 1991), renal tubular epithelial cells (Gomez - Villamondos *et al*, 1995) and neutrophils (Casals *et al*, 1984; Carrascosa *et al*, 1991). The virus is very resistant to inactivation in the environment (Scott, 1965) and serum may remain infectious for as long as 18 months when kept at room temperature (Montgomery, 1921). The effects of temperatures and pH on the virus had been

extensively studied (Plowright and Parker, 1967). ASFV survives standard heat treatment of serum used in the laboratory. The virus may also survive extremes of pH. Since ASFV possesses the ability to survive outside the host, it may persist in raw unprocessed meat for several weeks or months (Sanchez-Botija, 1982), especially in frozen meat. Cooked or canned hams contain no virus provided they are heated to 70$^0$C throughout. ASFV in biological products can be inactivated by exposure to gamma radiation (McVicar *et al*, 1982). Disinfectants containing the substituted phenol compound (o-phenyl-phenol) had been reported to be very effective in killing the virus (Hess, 1971).

## 2.4 **Structure of the Viral Particle**

ASF virions are icosahedral in morphology and are assembled in the cytoplasm of infected cells (Breese and DeBoer, 1966). The virus particles consist of a nucleoprotein core about 80nm in diameter surrounded by a lipid membrane covered by a capsid. Extracellular virion have an external envelope, derived by budding through the cell membrane, and are about 200nm in diameter (Breese and DeBoer, 1966; Carrascosa *et al*, 1984; Moura-Nunes *et al*, 1975). The external envelope does not seem to be essential for infectivity (Moura-Nunes *et al*, 1975). The capsid has a periodic structure and consists of a hexagonal arrangement of capsomeres which appear as hexagonal prisms 12nm long (Almeida *et al* 1967; Carrascosa *et al*, 1984).

Estimates of the number and molecular weights of ASF virion proteins vary considerably. Earlier reports estimated that there were 5 or 14 proteins (Black

and Brown, 1976), whereas more recently 24 intracellular virion proteins (Schloer, 1985; Tabares *et al,* 1980) and 34 extracellular virion proteins (Carrascosa *et al,* 1985) have been identified. Of the 34 extracellular virus proteins, 12 are probably of host cell origin. The extracellular virus contains no glycoprotein and no proteins with molecular weight above 150.000 daltons (Carrascosa *et al,* 1985).

## 2.5 Structural Proteins

There are 11 ASFV genes encoding proteins present in the virus particle. Most of them have been mapped by hybridization with degenerated oligonucleotide probes deduced from either the amino terminal sequence of the purified protein or from the sequence of some internal peptide. At least six of the structural proteins are encoded by only two genes, CP2475L and CP530R (Yanez *et al,* 1995). These genes code for polyprotein precursors which are orderly cleaved at the specific sequence Gly-Gly-X, giving rise to mature proteins (Yanez *et al,* 1995). Thus CP2475L encodes a polyprotein of 220KDa which, after cleavage, generates four major structural proteins: P150, p37, p14, and p 34 ((Yanez *et al,* 1995). CP530R is also processed after translation, giving rise to at least two virion proteins (Yanez *et al,* 1995). It has been suggested that, similar to what has been described for other viruses, the ordered cleavage of CP2475L (and probably that of CP530R) could have an important role in viral morphogenesis (Yanez *et al,* 1995).

Four ASFV genes encode structural proteins with a putative transmembrane region. 061R (Alcami *et al*, 1992), KP177R (Camacho and Vinuela, 1991) and EI83L (Rodriguez *et al*, 1994) code for proteins P12, P22 and P54 respectively. In infected cells, P22 can be transiently detected in the plasma membrane early in infection (Camacho and Vinuela, 1991), while P12 and P54 are late proteins localized in viral factories (Alcami *et al*, 1992; Angulo *et al*, 1993, Rodriguez *et al*, 1994). P12, P22 and P54 are located in the outer layers of the virus particle (Carrascosa *et al*, 1991; Camacho and Vinuela, 1991; Rodriguez *et al*, 1994). P12 is a virus attachment protein that seems to be active as a homodimer of 17KDa named P17 (Carrascosa *et al*., 1991; Angulo *et al*, 1993).

Three ASFV genes code for structural proteins with demonstrated or putative DNA binding capacity. Open reading frames (ORF) A104R (a Malawi isolate) encodes a protein with sequence similarity to bacterial histone-like proteins (Neilan *et al*, 1993). K78R encodes a protein of 10KDa, named P10, which is able to bind both single-stranded and double-stranded DNA with similar affinity (Munoz *et. al*, 1993). DNA binding by P10 is not sequence-specific, thus resembling proteins involved in DNA packaging or condensation and single stranded DNA binding proteins (Munoz *et al*, 1993). P10 seems to bind DNA in a co-operative manner (Munoz *et al*, 1993), something characteristic of single stranded DNA binding proteins (Chase and Williams, 1986). The function of this protein is not yet known, but it may be involved in ASFV transcription (Salas *et*

*al*, 1988). The third gene ORF E120R codes for structural protein P14.5, with DNA binding capacity.

Two other structural proteins, P72 and P11.5 are encoded by ORFs B646L (Lopez-Otin *et al*, 1990; Cistue and Tabares, 1992) and A137R (Alcami *et al*, 1993), respectively. Interestingly, the amino acid sequence of P72 shares some similarity with major structural proteins from iridoviruses (Schnitzler and Darai, 1993) and cholera virus PBC-1. ASFV P72 is a major virion protein produced late in infection (Carrascosa *et al*, 1985; Cistue and Tabares, 1992). Binding experiments with a collection of monoclonal antibodies specific for different ASFV proteins have shown that P72 is very conserved antigenically when different isolates and clones are compared (Garcia-Barreno *et al*, 1986). This, together with the fact that it is very immunogenic in natural infections (Tabares *et al*, 1980), has led to the use of recombinant P72 in a diagnostic test for African swine fever (Freije *et al*, 1993). On the other hand, P11.5 is a late protein identified as a minor component of virus particles (Alcami *et al*, 1993).

## 2.6 **Enzyme Activities**

ASF virus particles contain a number of enzymes including a DNA - dependent RNA polymerase which can transcribe ASF viral DNA in vitro (Kuznar *et al*, 1980) and resembles vaccinia virus RNA polymerase kinetically and in its pattern of enzyme inhibition (Salas *et al*, 1981). In addition, ASF virion contain enzymes involved in the $5^1$ and $3^1$ end modifications of mRNAs (Salas *et al*,

1981), a protein kinase (Polatnick *et al*, 1974) and one or two nucleoside triphosphate phosphohydrolases (Kuznar *et al*, 1981).

## 2.7 Nucleic Acid Metabolism

ASFV encodes three key enzymes for the synthesis of deoxynucleotide precursors for DNA replication, thymidine kinase (Martin-Hernandez and Tabares, 1991), thymidilate kinase (Yanez *et al*, 1993a) and ribonucleotide reductase (Bournsnell *et al*, 1991). The virus also codes for a deoxyuridine triphosphatase (Dixon *et al*, 1994) which presumably lowers the d UTP concentration in the infected cell to minimize misincorporation of d UTP into DNA (Shlomai and Kornberg, 1978). Increased thymidine kinase (Polatnick and Hess, 1970) and ribonucleotide reductase (Cunha and Costa, 1992) activities have been observed in ASFV-infected cells, and the former activity is dispensable for ASFV infection in tissue culture (Rodriguez *et al*, 1994). Vaccinia virus (Goebel *et al*, 1990) and Herpes simplex virus (McGeoch *et al*, 1988) encode similar sets of enzymes for nucleotide metabolism, suggesting that this could be a general strategy to overcome the allosteric or cell-cycle regulation of the corresponding cellular enzymes or to target the enzymatic activity to the most appropriate subcellular location for the viral infection.

ASFV also encodes enzymes for DNA replication. Genes G1207R ( Martin *et al*, 1994) and NP419L (Yanez and Venuela, 1993) code for an $\alpha$ - like DNA polymerase and an ATP-dependent DNA ligase, respectively, which probably

corresponds to the DNA polymerase (Polatnick and Hess, 1972) and DNA ligase (Yanez and Vinuela, 1993) activities detected in ASFV-infected cells.

ASFV particles carry a DNA-dependent RNA polymerase which is not sensitive to α- amanitin (Kuznar *et al*, 1980). The sequencing of NP 1450L and EP 1242L, two ASFV genes homologous to those encoding the two largest subunits of eukaryotic RNA Polymerase II and vaccinia RNA polymerase, suggested that ASFV also encodes a multisubunit enzyme (Yanez *et al*, 1993b).

ASFV particles contain enzymatic activities for mRNA capping and polyadenylation (Salas *et al*, 1981) and a previous study showed that PN868R is a guanylyltrasferase (Pena *et al*, 1993).

## 2.8 Immune Response to ASFV by Host

Antibodies to ASFV can first be detected at 3 - 4 days after infection, by radio immunoassy (Wardley and Wilkinson, 1980) and ELISA (Sanchez-Vizcaino *et al*, 1983). These antibodies are of the IgM class (Wilkinson, 1989). Antibodies can be demonstrated by complement fixation, immunopreciptation and immunofluorescence from 7 days after infection (Coggins, 1974). Virus replication and dissemination through the animal occur rapidly and both virus and antibody are present in the blood throughout the course of acute disease (Wilkinson, 1989). There is however no evidence that these antibodies are capable of neutralizing virus infectivity (DeBoer, 1967; DeBoer *et al*, 1969). In spite of the absence of neutralizing antibodies, pigs infected with the less virulent isolates may recover or progress to chronic disease (Wilkinson, 1989).

Studies on the pathogenesis of acute infections have shown that many of the cells involved in the development of an immune response are destroyed or removed from the circulation either as the direct result of virus replication or by indirect means (Wilkinson, 1989). The function of both B and T lymphocytes is affected; the effect on these functions, either stimulatory or inhibitory, appears to depend on the virulence of the isolate (Wardley and Wilkinson, 1980; Sanchez-Vizcaino *et al*, 1981). It is till not clear whether the development of the immune response by pigs infected with virulent strains is responsible for their recovery or their death due to an autoimmune phenomenon (Wardley and Wilkinson, 1980a).

Since neutralizing antibody is absent in pigs infected with ASF, the role of other types of antibodies and of different effector mechanism possibly involved in ASF pathogenicity, immunity or recovery has been investigated by several groups of workers. Schlafer *et al*, (1984) and Wardley *et al*, (1985) demonstrated that passively administered antibody can reduce the level of virus replication and the severity of disease in pigs infected with virulent isolates.

Antibodies effective in antibody dependent cell-mediated cytotoxicity (ADCC) assays, in which only neutrophils act as effector cells, are present from 13 days post infection (Norley and Wardley, 1983). While ADCC reaction reduces the virus yield of infected cells in vitro, pigs with acute disease generally die before these antibodies are detected (Wilkinson, 1989).

Complement and antibody from infected pigs lyse cells through the classical pathway of activitation (Norley and Wardley, 1982). Antibody binding

to cell surface antigens could be detected 5 - 7 days after virulent virus infection (Wilkinson, 1989). It does not bind complement until 14 - 15 days; it is therefore probably produced too late to prevent direct cell destruction and high virus yield in infections caused by virulent virus replication (Wilkinson, 1989). However, complement - mediated antibody lysis of infected cells was shown to occur before virus release in vitro. Complement-mediated antibodies induced buy some isolates do not necessarily produce lysis of cells infected with other isolates (Wilkinson, 1989). Such isolate specificity has also been demonstrated in pigs passively immunized with antibody. The ADCC reaction, however, shows no such specificity (Wardley *et al*, 1985). It was concluded (Wilkinson, 1989) that complement-mediated lysis may be effective in reducing virus replication, whereas ADCC may provide protection against clinical signs and death. He further added that in both mechanisms, antibody is probably produced too late to be important in the virulent virus infection.

It has been shown (Norley and Wardley, 1984) that cytotic lymphocytes are developed by 6 -7 days after infection and that they may play a role in the play a role in the pathogenesis of the disease.

In conclusion, whatever mechanisms are involved in their survival, pigs which recover are resistant to challenge with homologous virus (Hess, 1971; Ruiz-Gonzalvo *et al*, 1983) and are generally susceptible to challenge with heterologous isolates (Hess, 1971; Thomson *et al*, 1979).

Chronic disease has been produced experimentally by challenging pigs with virulent virus after recovery form infection with attenuated or avirulent virus. These pigs may have high levels both of antibodies detectable by complement fixation and immunoprecipitation (Hess, 1981) and of gamma globulin in the blood (Pan *et al,* 1970). They can produce neutralizing antibody to viruses other than ASF (DeBoer, 1967).

Delayed hypersensitivity is developed to ASF virus antigens and specifically sensitized lymphocytes may be present in the circulation for as long as 27 months (Shimizu *et al,* 1977). Chronic disease is usually fatal and it has been suggested that the lesions may be immunopathological (Hess, 1971; Coggins, 1974); antigen - antibody complexes have been demonstrated in the necrotic lung lesions in chronically affected pigs (Pan *et al,* 1975), and the necrotizing pneumonia cannot be accounted for by the cell destruction produced by the virus alone.

Maternal antibodies in recovered sows provide passive protection to piglets when challenged with fully virulent virus up to 7 weeks after birth (Schlafer *et al,* 1984a ). This protection is not complete, although it reduces the level of virus replication and the severity of clinical disease (Wilkinson, 1989). Piglets receiving colostral antibody have a delayed onset of clinical signs a brief period of fever, low levels of viraemia and a significantly higher survival rate than piglets fed on normal colostrum (Wilkinson 1989).

## 2.9 Immune Response Modulation

ASFV encodes a number of proteins potentially able to interfere with the host immune response to the viral infection. Thus, the virus codes for a protein similar to the T-cell adhesion molecule, $CD_2$ (Rodriguez *et al*, 1994; Borca *et al*, 1994). $CD_2$ is an adhesion molecule located in the cytoplasmic membrane of T-cells and natural killer cells (Beyers *et al*, 1989; Bierer and Burakoff, 1989). PEP402R, the CD2 homologue encoded by ASFV, is a membrane protein responsible for the adhesion of erythrocytes to infected cells in tissue culture (Rodriguez *et al*, 1994; Borca *et al*, 1994). The adhesion capacity of $CD_2$ mediates antigen-specific and antigen independent T-cell activation and natural killer cell activity (Beyers *et al*, 1989; Bierer and Burakoff, 1989) and restricts complement mediated lysis of erythrocytes and leukocytes (Whitlow *et al*, 1990). The action of these effector cells on ASFV infected cells may be blocked by the presence of the ASFV $CD_2$ in the plasma membrane (Rodriguez *et al*, 1994). The rosetting of erythrocytes around ASFV-infected cells has been observed in vivo (Sierra *et al*, 1991), and it could be speculated that the accumulation of RBCs may cause a steric hindrance to the action of immune effector cells on the surface of infected monocytes and macrophages.

## 2.10 Antigenic and Immunological variation in ASF Viruses.

Immunological diversity amongst strains of ASFV has been challenged and occasionally even exposure to heterologous strain (De Tray, 1960, Hess, 1971, Malmquist, 1963), the immunity in some cases was however not absolute and

or enduring. Malmquist (1963) found that attenuated 1954 wart-hog isolate (Hinde 1) failed to protect two pigs against a second wart-hog population. He also showed that it was haemadsorbing virus of the challenge type which predominated in the blood of pigs which were inoculated with a heterologous strain.

Unfortunately, in the virtual absence of neutralizing antibodies, the only way to investigate immunological variation among ASF viruses is to challenge the immunity of recovered animals, there was and is no serological correlate of resistance, in spite of early indications from Malmquist's work (Malmquist, 1963) that strains which were similar in their haemadsorption antigens did cross-protect, while those that differed in this respect failed to do so. It soon became obvious that recovered resistant pigs often had no serum inhibitor of homologous or heterologous virus haemadsorption (Coggins, 1968, Coggins, 1974, Thomson *et al*, 1979). Furthermore, in South Africa, pigs which had recovered from infection with a naturally occurring, plague-purified and non-haemadsorbing strain of much reduced virulence from Zaire were resistant to heterologous challenge with three different haemadsorbing isolates (Thomson *et al*, 1979). In addition, some of these resistant pigs developed haemadsorption-inhibiting (Had I) antibodies following challenge and the haemadsorbing virus was recovered from the spleen of an animal exposed intranasally. It was concluded that haemadsorption was neither a marker of virulence nor could its inhibition be used to determine significant immunological differences (Thomson *et al*, 1979).

## 2.11 ASFV Taxonomic Position

Due to its intermediate characteristics between poxviruses and iridoviruses, ASFV has recently been excluded from family Iridoviridae (Brown, 1986). ASFV and poxviruses have the necessary enzymes for early mRNA synthesis in the virus particle, produce polyadenylated mRNA, and have a similar DNA structure with hairpin loops and terminal inverted repeats (Vinuela, 1987). On the other hand, ASFV and iridoviruses share the icosahedral morphology, seem to lack structural glycoproteins, depend on the presence of the cell nucleus for virus multiplication (Vinuela, 1987), and may have nuclear and cytoplasmic stages of DNA replication (Goorha, 1982).

The availability of the complete sequence of the ASFV DNA (Yanez *et al*, 1995) allows a comprehensive comparison with that reported for the vaccinia virus genome (Goebel *et al*, 1990). Most of the genes close to vaccinia DNA ends are transcribed outwards, while this is not true for the right DNA end of ASFV. Another difference is the abundance of repeated sequences out of the TIR (terminal inverted repeats) in the ASFV genome, while in vaccinia virus they are scarce (Smith *et al*, 1991). With very rare exceptions, the only proteins with amino acid sequence similarly between ASFV and vaccinia are enzymes of nucleotide and nucleic acid metabolism. Whenever phylogenetic trees or cluster dendograms have been deduced from multiple alignments of protein sequences, the ASFV and pox viral enzyme do not fall into the same groups (Bournsnell *et al*, 1991; Kletzin, 1992).

ASFV may also regulate infected cell adhesion through the CD2 nomologue while vaccinia does not possess a similar gene. In addition, the DNA sequences regulating transcription in ASFV (Almazan *et al*, 1992; 1993) seem to be different from those reported for vaccinia virus (Moss, 1990). Taken together, these reasons seem to indicate that ASFV and vaccinia have similar genomic structures and possess equivalent genes for nucleotide and nucleic acid metabolism, but most of their genomic information does not seem to be related.

The DNA sequence data available from iridoviruses are scarce, but they allow some comparisons with ASFV. The genomes of the prototype iridoviruses are circularly permuted and redundant (Willis, 1990). Like the ASFV genome, they contain repetitive elements (Fischer *et al*, 1994; Schnitzler *et al*, 1990). The major capsid proteins of iridoviruses has some sequence similarity to one of the major structural proteins of ASFV, P72 (Schnitzer and Darai, 1993) and both chilo iridescent virus (Birnbaum *et al*, 1994) and ASFV encode a protein similar to the baculovirus apoptosis inhibitor IAP. However, the sequences reported for the chilo iridescent virus RNA polymerase largest subunit (Schnitzler *et al*, 1994b), helicase and 8-hydroxy-d GTPase and that of the thymidine kinase of fish lympholystis disease virus (Schnitzler *et al*, 1991), are not especially related to the corresponding proteins of ASFV. Finally, chilo iridescent virus encodes a high mobility group protein homologue, but there is no such similar protein in ASFV. Thus, DNA sequence data confirm previous biochemical and morphological results about the intermediate characteristic of ASFV between iridoviruses and poxviruses

and showed that the three viral groups have additional properties of their own. Hence no conclusive reason to alter the current notion of ASFV as the only known member of an as yet unnamed separate genues - African swine fever - like viruses in the family poxviridae (Murphy *et al,* 1995).

## 2.12 Clinical Disease and Pathology

Historically, the disease was peracute. Montgomery (1921) recorded a mortality rate of 98.9% in outbreaks involving 1366 swine. Recently, however, many gradations of the disease are seen and for convenience ASF is now normally discussed under four headings, peracute, acute, subacute and chronic disease (Hess, 1981).

In the peracute form, the disease manifests as haemorrhagic fever with very high mortality usually close to 100% especially when African isolates are incriminated (Montgomery, 1921, De Tray, 1963). Pigs can die without showing any signs of illness and although widespread haemorrhages are a common post-mortem finding, gross lesions may be minimal.

With the acute form, pigs show a high temperature of about 40 - 42°C for 1 - 2 days whilst still behaving normally; sometimes the pyrexia drops to subnormal temperature in moribund animals. Thereafter, they then become anorexic and reddened or cyanotic areas appear at the extremities and some animals show respiratory signs, vomiting and blood-tinged diarrhoea. Sometimes mucopurulent discharges from the eyes and nose, conjunctivitis, vomiting, bleeding from the nose, and rectum. Death then ensures within 7 - 10 days of

clinical signs. On postmortem examination, there may be haemorrhages in almost every organ, particularly visceral lymph nodes, with an excess of natural fluids in body cavities and spaces. The distribution and frequency of those lesions are very variable and many of them also occur in other swine diseases. Pregnant sows usually abort (Schlafer and Mebus, 1984).

The subacute form of the disease has been described in Spain and Portugal (Sanchez-Botija, 1982). With this form, animals can show a persistent or fluctuating temperature response for up to 20 days, during which time some pigs can remain alert and in good condition, while others display the symptoms described above but in a less severe form. Mortality rates vary and often seem to depend on host factors such as breed, age, and general health of the pig (Hess, 1981). The pathogenesis of such isolates is similar and postmortem signs include features listed for the acute form of the disease. Recent isolates which have fallen into this group have come from the Iberian Peninsular, Dominican Republic, Brazil and Malta (Wilkinson *et al*, 1983).

Chronic form occur in places where ASF has become enzootic in domestic pigs. Clinical signs are variable and may persist for many months. Symptoms include pneumonia, stunting, emaciation, arthritis and skin ulcers (Hess, 1981). Cyclic pyrexia is not uncommon. When animals die, haemorrhages may again be the dominant post mortem lesions, although fibrinous pericarditis and pleuritis accompanied by severe pneumonic changes are also common (Hess, 1981). Hypergammaglobulinaemia is often observed (Pan *et al*, 1970).

It is difficult at postmortem to differentiate ASF from classical swine fever (Hog cholera) (Maurer *et al*, 1958). This therefore emphasizes the necessity of laboratory diagnosis.

Experimental studies have shown that virus replicates primarily in cells of the reticulo-endothelial system (Colgrove *et al*, 1969; Enjuanes *et al*, 1977, Greig, 1972; Wardley and Wilkinson, 1977; Wilkinson and Wardley, 1978) and severely affects the total and differential white blood cell picture (Wardley and Wilkinson, 1977; Wardley *et al*, 1977) with a marked effect on subpopulations of lymphocytes (Wardley and Wilkinson, 1980a). Morphological studies on its replication in macrophages suggest that virulent and attenuated isolates behave differently which may result in functional changes directly affecting the immunological response of the pigs. Experiments using virulent isolates have indicated that they have a very marked stimulatory effects on cells of the immune system (Pan *et al*, 1970) and that lesions in the pig may be caused by an auto-immune type phenomenon (Pan *et al*, 1975; Wardley, 1982). It has been suggested that avirulent isolates are non-pathogenic because they suppress the immune response (Wardley, 1982). Although this and other work has implied that ASF virus induces a cell-mediated response, there is no direct knowledge of T-cell responses (Sanchez- Vizcaino *et al*, 1981; Tabares *et al*, 1980). This evidence suggests that the demise of infected animals is brought about by the indirect effects of virus multiplication on a number of systems including

complement and platelets (Slauson and Sanchez-Vizcaino,1981) rather than by the direct cytolytic effect which the virus has on many cell types.

## 2.13 Diagnosis

Laboratory studies are essential to establish a correct diagnosis of ASF due to the great similarity of ASF clinical signs and necropsy lesions with other haemorrhagic pig diseases (hog cholera, erysipelas or septicemic salmonellosis). As in other virus diseases, the ASF laboratory diagnosis can be based on the demonstration of infectious virus, viral antigens, viral DNA or specific antibodies. A wide variety of laboratory tests are already available for both ASF virus and antibodies detection (Sanchez-Vizcaino, 1986).

## 2.14 ASF Virus Detection

Out of the several techniques adapted for the identification of ASF virus, the most convenient, safe and frequent techniques used at present are: direct immunofluorescence (Bool *et al*, 1969) and hemadsorption test (Malmquist and Hay, 1960).

Direct immunofluorescence is a very fast and economic test with high sensitivity in the acute form of ASF. Infact it is the test recommended to be used first under the suspicion of ASF. Direct immunofluorescence is based on the demonstration of viral antigen on impression smears or frozen tissues section from spleen, lung lymphatic nodes or kidney, through a conjugated immunoglobulin against ASF virus.

The hemadsorption test is a universal technique used for ASF virus detection due to its sensitivity and specificity. This test is usually performed to confirm any new outbreak and also in case some other tests have been negatives. This test is based on the hemadsorption characteristic of ASF virus, which are induced when the virus infects macrophages and the attachment of the erythrocytes on the membrane of infected cells form a characteristic rosette around the macrophages, before the cytopathic effects appear (Malmquist and Hay, 1960). It has been observed (Sanchez-Botija, 1982) that although hemadsorption is the most sensitive test for ASF virus identification, there are a small number of field strains showing only cytopathic effect without producing the hemadsorption phenomenon. These strains are identified using the direct immunofluorescence test on the sediments of these cell cultures.

## 2.15 ASF-DNA Detection

Since its first reported use 1985 (Saiki *et al*, 1985), PCR has revolutionized recombinant DNA technology.

Polymerase chain reaction (PCR) nas been developed for the detection of ASF virus genome, using a primer from a highly conserved region of the viral DNA, to detect a wide range of ASF isolates belonging to all the known virus genotypes including both non hemadsorbing viruses and low virulence ones. Principle of the test is an enzymatic method of making multiple copies of a pre-selected segment of DNA (Campbell, 1991) This amplification process is achieved with two synthetic oligodeoxynucleotide primers (A and B), a thermostable DNA

polymerase and the four deoxy-ribonucleoside triphosphates acting on the template DNA. The first primer (A) matches one of the strands at one end of the segment, while the second primer (B) matches the other strand at the other end. When the template DNA is denatured by heating and then cooled, the primers anneal to their targets on the single- stranded templates. The DNA polymerase then extends the primers at the $3^1$-ends. Since the $3^1$-ends of the primers point towards each other, repeated cycles of heating and cooling lead to a chain reaction, an exponential synthesis of many copies of the specific segment bounded by the two primers. PCR is particularly useful for virus DNA identification from tissues with poor conservation even if they have undergone putrefaction or in those case where the virus has been inactivated (Saiki *et al,* 1991). It is an excellent and rapid technique to be included in ASF diagnosis test. However, it is expensive and technically demanding.

### 2.16 Antibody Detection

Several techniques have been adapted to ASF antibody detection: Complement fixation test (Cowan, 1961), indirect immunofluorescence (Bool *et al,* 1969), immuno-electroesmophoresis (Pan *et al,* 1972), enzyme linked immunosorbent assay (Sanchez-Vizcaino *et al,* 1979, 1983), Radio immunoassay (Wardley and Wilkinson,1980) and immunoblotting assay (Pastor *et al,* 1987). Yet, only indirect immunofluorescence, immunoelectroosmophoresis (IEOP), ELISA and immunoblotting are the most frequently used.

The indirect immunofluorescence is a fast technique with high specificity and sensitivity for the detection of ASF antibodies from either sera or tissue exudate (Sanchez- Botija *et al*, 1970).

At present, the ELISA test is the most useful method for large scale ASF serological studies. It is based on the detection of ASF antibodies bound to the viral proteins by addition of protein A, which is conjugated with an enzyme that produces a visible colour reaction when it reacts with the appropriate substrate.

The immunoblotting test is a highly specific, sensitive and easy to interpret technique which has been successfully used as an alternative method to indirect immunofluorescence for low or doubtful ELISA sera confirmation (Arias and Sanchez-Vizcaino, 1992).

IEOP is a rapid and accurate method with extreme sensitivity and superior to the complement fixation and agar gel diffusion precipitation test in detecting antibody against ASF virus (Pan *et al*, 1972).

**2.17 Treatment**

At present, no treatment or effective vaccine against ASF is available. Since when the first live-attenuated vaccine was used in Portugal (Manso-Ribeiro *et al*, 1963), many efforts have been made in this area with unsatisfactory results. Inactivated vaccine does not produce any protection, and live-attenuated vaccine protects some animals against the challenging infection with the homologous virus, but some of these animals becomes carriers and develop chronic lesions, especially if vaccination is made on large scale (Manso- Ribeiro *et al*, 1963;

Sanchez-Botija, 1963). Other studies have shown that serum from resistant pigs to homologous and some heterologous ASF virus inhibits the infection of different but related heterologous virus *in vitro* (Ruiz Gonzalvo *et al*, 1983). However, in the classical concept of neutralization, ASF specific antibodies have never been demonstrated to neutralize virus.

## 2.18 **Prevention**

The lack of a vaccine against ASF is probably the single most important fact which makes it potentially the most damaging infection which the international swine industry has to face. Epizootiological studies have shown that the most frequent source of ASF contamination was the garbage of international airports or ports. Therefore, all food leftover from planes and ships should be incinerated.

In infected European areas as Sardinia where the disease is enzootic and where mild or inapparent clinical signs can be observed, the most important aspect of ASF prevention include the control of animal movements and extensive serological surveys to detect carrier pigs.

In endemic areas of Africa, the most important factor is to control the natural reservoirs and to prevent its contact with domestic pigs. A rapid differential laboratory diagnosis is required in case of any suspicious haemorrhagic pig disease. It is important to also remember that low virulence ASF strains do not produce great lesions.

## 2.19 Update of the Situation of ASF in Nigeria

ASF outbreaks were first reported in Ogun, Osun and Lagos states in late December 1998. Although the Osun outbreak were said to have started in April, 1998, retrospective evidence from sera seem to indicate that the disease was present in the area as far back as late 1997 (Majiyagbe, 1999). The disease spread beyond these states to involve several other states namely: Delta, Enugu, Rivers, Akwa-Ibom, and Ondo in the Southern as well as Kaduna, Benue and Plateau in the Northern part of the country. However, the disease may also be present undetected in other states due to inadequate or non-existing surveillance.

Benue state that has about 21.5% of the country's pig population experienced the most devastating effects of the epizootics. By the end of September 1988, about 79, 748 pigs were affected and 61, 298 of them died (EMPRES, 1998).

The individual state reports on outbreak status are presented in Appendix V.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Pigs:

### 3.1.1 Jos abattoir

The pigs slaughtered at the Jos abattoir provided the source for both sera and tissue samples used from Plateau state. The pigs consisted of local black, white or mixed coloured types. These pigs are usually kept under poor conditions of husbandry and obtain much of their food by scavenging in the streets during the day and night. The butchers buy these pigs from dealers and pig owners within Plateau state while some come from the southern part of Kaduna state.

### 3.1.2 Kaduna state abattoir

Pigs slaughtered at the slaughter slabs in 4 local government areas of southern part of Kaduna state, namely, Zangon Kataf, Kachia, Jema'a and Kaduna south, were the source for both sera and tissues samples used form Kaduna state.

## 3.2. Sample Collection:

Blood sampling was carried out between April, 1999 and February, 2000. The animals were slaughtered usually every Saturday morning for Jos abattoir, and about twice a week for the areas considered in the Southern part of Kaduna State.

Five hundred and ninety (590) serum samples were collected from Jos abattoir and 195 serum samples collected from four (4) Local Government Areas

of Southern Kaduna namely: Zangon Kataf, Kachia, Jema'a and Kaduna South. About 5.0ml of blood was collected from each slaughtered pig into clean McCartney/Bijou bottle. The samples were then arranged on a slant and allowed to clot. The sera was separated from the rest of the blood components into another clean bottle. The separated sera transported to the laboratory on ice were further centrifuged and stored at -20°C until used.

In addition, spleen and lymph nodes were collected from 20 pigs at the Jos abattoir and from six (6) pigs from the Southern part of Kaduna State. These tissues were kept in tight covered universal plastic containers and transported to the laboratory on ice for viral antigen detection and DNA extraction for polymerase chain reaction (PCR) test.

## 3.3 Methods

### 3.3.1 Indirect Enzyme Linked Immunosorbent (I-ELISA)

Antibody detection was carried out using I-ELISA based on methods used in Office of International Epizootics (O.I.E.) manual of Standards (Sanchez-Vizcaino *et al*, 1979) and INIA-CISA, Madrid, Spain (Sanchez-Vizcaino *et al*, 1983). Biological reagents used were supplied by INIA. All the biologicals, reagents and buffers used for the test were prepared as shown in appendix 1.

**Test Protocol**

Five litres of VIRKON(R) disinfectant at 1:200 was prepared and divided into two for the disposal of all used plastic tips and glass wares. Stored serum samples were allowed to thaw at room temperature and details of all the sera

to be tested were recorded on the ASF ELISA work sheet as shown in appendix II. Flat bottomed Nunc Maxisorp ELISA microplates supplied in the standard ELISA kit supplied by INIA laboratory, Madrid, Spain, were used.

ASF virus antigen was diluted to a pre-titrated concentration of 1:1600 in coating buffer (pH 9.6). One hundred microlitres (100 µl) of the diluted ASF virus antigen was dispensed into all the wells of each plates using a multichannel micropipette and incubated overnight in sealed containers at +4°C. The following day, all the contents of each coated plates were discarded into the disinfectant solution and each plate was washed at least six times in wash buffer (pH 7.2) and blot dried on towels. The washings were to remove all unbounded antigens. Test and control sera (ASF positive and ASF negative serum) were diluted 1:30 in PBS/Tween 20 (pH 7.2) solution and 100 µl of each diluted serum was dispensed into duplicate plate wells and incubated for 1 hour at 37°C on incubator shaker.

Again, all the plates were washed six times using washing buffer and blot dried on towels.

One hundred microlitres (100 µ) of protein-A horseradish peroxidase conjugate, diluted in PBS/Tween 20 (pH 7.2) solution at 1:2000 was dispensed into each well and incubated for 1 hour at 37°C on incubator shaker. The diluted conjugate remaining was left in a 20ml bottle, which was later used to check if there was colour reaction with the chromogen/ substrate solution.

Again, all the plates were washed six times using wash buffer and blot dried on towels.

At this point the ELISA reader was switched on to allow it warm up for about 30 minutes before plates were read.

Just before the addition of OPD to the ELISA Plate, $H_20_2$ at the rate of 4ul per ml of OPD was added and mixed thoroughly. One hundred microlite (100 µl) of this substrate solution was added to each plate well and incubated in the dark at room temperature for 20 minutes without shaking for colour development. At the same time a new flat bottom NUNC maxisorp ELISA microplate was used as a blanking plate by dispensing 100 µl per well of the chromogen/substrate solution to only the first column of the plate.

All reactions were stopped by the addition of 100 µl of $1MH_2SO_4$ to all the wells.

All optical densities of each plate well was measured by an ELISA plate reader, connected to an IBM computer, at 492nm.

The colour intensity developed was proportional to the amount of specific antibody bound to the antigen. A serum was considered ASFV antibody positive if it has an optical density (OD) value greater than the cut-off-point. However, if a serum has an OD value less than the cut-off-point, it was considered as ASFV antibody negative. Doubtful results were retested.

Cut-off OD value was calculated using the formula;

Cut. off = Mean OD negative serum x 1 + Mean O.D. positive serum x 0.2.

### 3.3.2. **Direct Immunofluorescence**

Antigen detection was carried out using direct immunofluorescence based on methods used in O.I.E manual of standards and INIA CISA, Madrid, Spain.

Reagents and buffers used for the direct immunofluorescence were prepared as shown in appendix III.

**Sample Preparation**

Twenty six slides were carefully removed from 100% ethyl alcohol and wiped clean using a lint-free cloth and a diamond pen was used to mark both position of slides and ASF positive and negative control tissue smears.

Starting with the ASF negative control tissue, using a pair of forceps and scissors the tissues were cut into a shape of a small cube and an impression smear was made on the slide. The same procedure was carried out for both ASF positive and test tissues.

All the tissue impression smears made were air dried at room temperature.

Between tissue samples, the forceps and scissors were dipped in alcohol and flamed so as to sterilize them and prevent carry-over of antigens or debris.

**Test Protocol**

Smears were then fixed in 100% acetone for 10 minutes in staining jar. Each slide was washed by gentle irrigation using wash bottle containing PBS and later transferred in staining jar filled with PBS for 3 minutes at room temperature.

Smears were then air-dried and a circle was drawn around each smear with nail vanish so as to limit the spreading of the conjugate.

One hundred to four hundred microliters (100 µl - 400 µl) of ASF antibodies conjugated to fluorescein at 1:50 dilution was added to each smear and incubated at 37°C for 1 hour in humidified container. Again, slides were washed by gentle irrigation using wash bottle containing PBS and later transferred in staining jar filled with PBS for 3 minutes at room temperature.

Thereafter, slides were air-dried and cover slips were mounted over each smears in 10% PBS-glycerol and viewed under x 100 lens of the fluorescence microscope. The ASF positive and negative control tissues were examined first followed by the test tissues. Tissues were considered positive if specific granular cytoplasmic fluorescence was observed.

### 3.3.3 **DNA Extraction and Polymerase Chain reaction (PCR).**

DNA extraction was done using Guanidinium thiocynate (GUSCN) method as modified by Bloom *et al,* (1990). Reagents and buffers used for the DNA extraction and PCR were supply by Ondersteeport Veterinary Research Institute, Pretoria, Republic of South Africa. They were prepared as shown in Appendix IV

**Sample preparation**:- A total of 10 tissue samples (comprising of 4 spleen and 5 lymph nodes that tested negative for ASFV antigens by direct immunofluorescence and 1 spleen that tested positive for ASFV antigens by direct immunofluorescence) were individually treated for DNA extraction and later tested in PCR.

One gram of each tissue sample was weighed and grounded separately in a sterile pestle and mortar containing sterile sand. A 1/10 dilution of each tissue sample was made in sterile water and transferred into a 20ml bottle and centrifuged at 2000rpm for 5 minutes. Then an aliquot of 1000 µl of the supernatant for each sample was taken and transferred separately into sterile 1.5ml eppendorf tube. They were all properly labelled and stored at -20°C until ready for use in the DNA extraction.

**DNA Extraction.**

An aliquot of 6ml of **L6** lysis buffer was transferred into a sterile 15ml tube. Silica was mixed thoroughly using a vortex mixer until it was completely in solution and 240 µl of this resuspended silica was then added to the lysis buffer in the clear tube (This is sufficient for DNA extraction from 10 samples).

At this time the tissue suspensions were allowed to thaw and 940 µl of the **L6**/silica mix was added to each tube (in the fume hood) using a clean filter tip each time. Each tube was mixed thoroughly using a vortex mixer and incubated at room temperature for 5 minutes with periodic shaking at intervals.

Then each tube was mixed properly using a vortex mixer for 5seconds and centrifuged for 15seconds using an ultracentrifuge The supernatant was then discarded off in 10M NAOH waste bottle. At this point, the nucleic acid are bounded to the silica pellet.

Nine hundred microlitres (900 µl) of **L2** wash buffer was added to each of the tubes containing the silica pellet (in the fume hood). Each tube was mixed

thoroughly using a vortex mixer until the silica pellet was completely dissolved. It was then centrifuged for 15 seconds to pellet the silica. Using a 1000 µl micropipette fitted to a fresh sterile tip for each tube, the supernatant was carefully removed and discarded into 10M NAOH waste bottle. Again this wash step was repeated using 70% alcohol and acetone respectively.

The silica pellets from each tube were then dried by placing the tubes (lids open) in an incubator preset to 56°C for 20 minutes until the pellets were completely dried.

Thirty microlitres (30 µl) of high purified liquid chromatography (HPLC) $H_20$ was added to each tube then vortexed and incubated at 56°C for 2 minutes after which the tubes were centrifuged for 10 minutes and the supernatant ( containing the eluted nucleic acids) were each transferred to new clean, RNAse – free 1.5ml eppendorf tubes. The tubes were properly labelled and stored at –20°C until ready for use.

**Polymerase Chain Reaction (PCR).** It was done based on the O.I.E. Manual of Standards for Diagnostic Tests and Vaccines (2000) .

**Test Protocol**

Preparation of master mix for PCR with final reaction volume of 25 µl was done (in 1.5ml eppendorf) as follows:-

| | 1X |
|---|---|
| Taq Polymerase (S.A Taq-MD(PTY)LTD) | 0.5 µl |
| 10X buffer+$Mg^{2+}$ | 2.5 µl |

| | |
|---|---|
| dNTPs (10mM) | 0.5 µl |
| primers pAS1 | 1.0 µl |
| Primer pAS2 | 1.0 µl |
| $H_20$ | 17.5 µl |
| DNA | 2.0 µl |
| Total | 25.0 µl |

For water control, 2.0 µl of $H_20$ was added in place of DNA.

The primer sequence comprised of:-

pAS1, $5^1$-ATG GAT ACC GAG GGA ATA GC -$3^1$

pAS2, $5^1$-CTT ACC GAT GAA AAT GAT AC -$3^1$

The PCR amplicon size was 278bp.

The individual DNA/master mix and the water control were each overlayed with 60 µl of mineral oil so as to prevent evaporation of the reaction mixture during PCR.

Thermocycler Programme was done by placing all the tubes in an automated PCR thermal cycler. The thermocycler was set to programme 5 (i.e. programme 5 linked to programme 6) which comprises the following.

i) Denaturation: 94$^0$C for 15 secs,

ii) Anneal: 62$^0$C for 15secs.,

iii) Extension:72$^0$C for 15seconds.

iv) Number of cycles: 30 cycles.

**Gel analysis of PCR products using 1.5% agarose in 1x TAE.**

0.75gm of agarose was weighed and transferred to a flask containing 50ml 1x TAE, to give a final concentration of 1.5%gel. The agarose was melted using gas burner. and then allowed to cool to about 50% and ethidium bromide was added to a final concentration of 0.5 µg/ml. The gel was then poured on the casting tray and all air bubbles were carefuily removed before the comb was inserted. The gel was transferred into the electrophoresis tank with the buffer slightly covering the gel. The gel was then loaded with 3.0 µl l of DNA marker and 2.0 µl of loading buffer in the first well.

Three microlitres (3.0 µl) of each PCR products were carefully removed from below the mineral oil and added to 2.0 µl of loading buffer, and were loaded separately into the rest of the wells as indicated on the label. The electrophoresis was allowed to run for 20 minutes at a constant voltage of 120 volts. At the end of the electrophoresis, the gel was observed using a U.V transilluminator and photograph of the gel was taken. Tissue was considered positive if it produced band which corresponded to the 278bp of the specific amplicon size of the primer.

2. 4 **Statistical analysis of Data.**

Data obtained in this investigation was statistically analysed using Chi-square test (Snedecor and Cochran, 1980) and Odds ratio (OR) (Thrushfield and Hinxman, 1981).

# CHAPTER FOUR

## Results.

### 4.1 Incidence of Antibodies to ASFV in pigs.

Results of prevalence of antibodies to ASFV in swine sera collected from some parts of Kaduna and Plateau states are shown in Table 4.1.

Out of a total of 590 serum samples collected at the Jos abattoir between April 1999 and February 2000, 15 (2.5%) had antibodies to ASFV, while 25 (12.8%) out of 195 samples from Kaduna state were also positve for antibodies to ASFV. Of the 195 pigs sampled from 4 local government areas of Kaduna state, Kaduna South local government area had the highest frequency of 29.4% while Jema'a had the least at 6%. The frequency varied from one local government area to another. Antibodies to ASFV was detected in all the local government area except Kachia. (Table 4.1).

The sex distribution of pigs that tested positive to antibodies to ASFV is shown in Table 4.2. The sex specific rates for males (7.4%) significantly differs from those of the females (3.6%) ($P<0.05$). Males were about 2 times more likely to have antibodies to ASFV than females.

### Detection of Antigens to ASFV in pigs.

Results of antigen detection to ASFV on swine tissues samples tested from some parts of Kaduna and Plateau states are presented in Table 4.3. All the tissues used for the antigen detection were swollen and haemorrhagic. 9 (45%) out of the 20 tissue samples tested from Plateau State were positive to ASFV

antigen, while 5 (83.3%) tested positive from Kaduna State (Table 4.3). Kaduna South local government area had the least antigen detection rate of 50% while the remaining 3 local government areas each had an incidence rates of 100% (Table4.3).

Tissues that tested negative for antigens to ASFV by DIF were again used to test for virus DNA genome ny the PCR. Results obtained by the PCR revealed that, out of a total of 9 tissues (4 spleen and 5 lymph nodes) that tested negative for antigens to ASFV by DIF, and 1 tissue (Lymph node) that tested positive for ASFV antigens by DIF, all the samples were positive for viral DNA expect 1 (spleen) from Plateau state. Fig 1 presented the photograph of the gel taken. Bands were produced in 9 out of the 10 extracted DNA tissues which corresponded to the 278bp of the specific amplicon size of the primer.

**TABLE 4.1: Frequency of Antibodies to ASFV in pigs sera from Kaduna and Plateau States.**

| State | Local Govt. Area | No sampled | No Positives | Percentage Positives(%) |
|---|---|---|---|---|
| Plateau | Jos North | M=195<br>F = 395<br>M+F = 590 | M=5<br>F = 20<br>M+F 15 | M=0.85%<br>F = 1.7<br>M+F 2.5 |
| Kaduna | Kaduna South | M= 30<br>F = 21<br>M+F = 51 | M=10<br>F = 5<br>M+F15 | M= 19.6<br>F = 9.8<br>M+F 29.4 |
| Kaduna | Jema'a | M= 35<br>F = 15<br>M+F = 50 | M= 3<br>F = 0<br>M+F 3 | M= 6.0<br>F = 0<br>M+F 6.0 |
| Kaduna | Kachia | M= 30<br>F = 21<br>M+F = 51 | M=0<br>F = 0<br>M+F 0 | M= 0<br>F = 0<br>M+F 0 |
| Kaduna | Zangon Kataf | M= 30<br>F = 13<br>M+F = 43 | M= 5<br>F = 2<br>M+F 7 | M= 11.6<br>F = 4.7<br>M+F 16.3 |
| | TOTAL SAMPLE TESTED | M = 310<br>F = 475<br>M+F = 785 | M = 23<br>F = 17<br>M+F = 40 | M = 7.4<br>F = 3.6 |

**Key: M = Male**

**F = Female**

**TABLE 4.2: Sex Distribution of Antibodies to ASFV in pigs from Plateau and Kaduna States.**

| Sex | Total No. samples | No. tested positive | No. tested negative | Sex Specific rate (%) | Odds Ratio |
|---|---|---|---|---|---|
| Males | 310 | 23 | 287 | 7.4 | 2.16 |
| Females | 475 | 17 | 458 | 3.6 | 1.0 |
| TOTAL | 785 | 40 | 745 | | |

**x2 = 5.721**

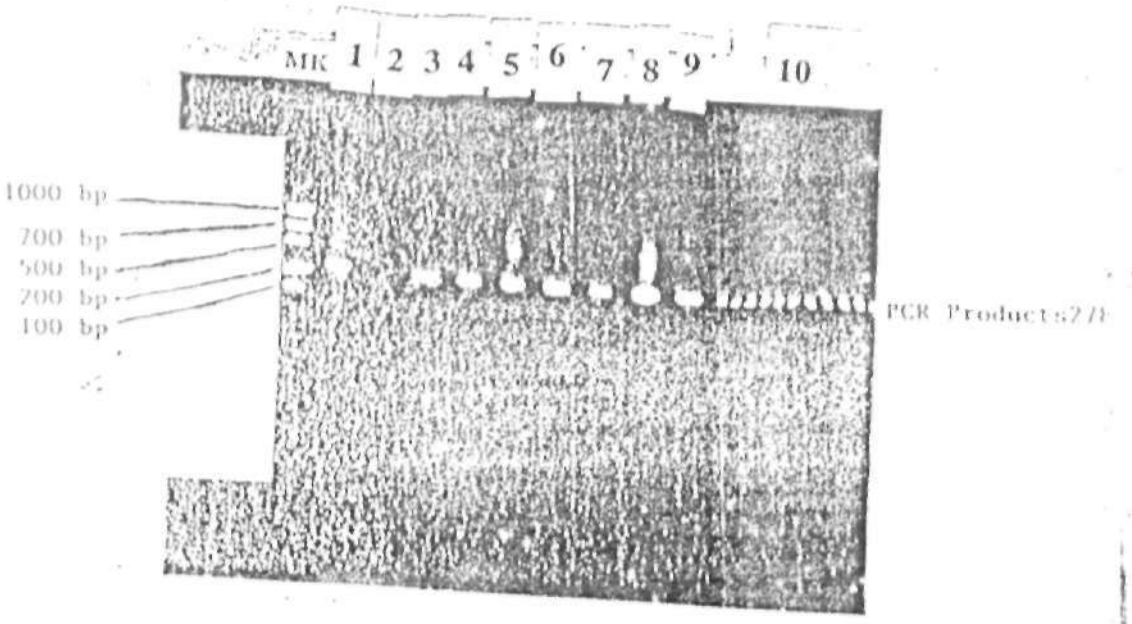
**DF = 1**

**p<0.05 significant**

**TABLE 4.3: Results of antigens to ASFV in swine tissue samples tested from some parts of Kaduna and Plateau state.**

| State | Local Govt. Area | Type of Tissue | No of Samples tested | No. Positive | Positive (%) |
|---|---|---|---|---|---|
| Plateau | Jos North | Lymph node | 10 | 6 | 46 |
| Plateau | Jos North | Spleen | 10 | 3 | 46 |
| Kaduna | Kaduna South | Spleen | 2 | 1 | 50 |
| Kaduna | Jema'a | Lymph node | 2 | 2 | 100 |
| Kaduna | Kachia | Lymph node | 1 | 1 | 100 |
| Kaduna | Zangon Kataf | Spleen | 1 | 1 | 100 |
| Total Tissue tested | | | 26 | 14 | 53.85 |

**Figure 1: PCR Analysis of DNA extracted from Pig tissue suspected of ASF**




Key:

MK = DNA Marker 100 – 1000 bp

1 = Spleen KD/South
2. = Spleen Jos North
3. = Spleen Jos North
4. = Spleen Jos North
5. = Lymph node Jos North
6. = Lymph node Jos North
7. = Lymph node Kaduna
8. = Lymph node Jos North
9. = Lymph node Jos North
10. = $H_2O$ -Negative Control

# CHAPTER FIVE

# DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS.

## 5.1 Discussion

A serological survey of swine sera from Plateau state and Southern part of Kaduna state for the presence of antibodies to ASFVwas carried out using Indirect – Enzyme Linked Immunosorbent Assay (I-ELISA). The overall prevalence rate of antibodies to ASFV for Plateau state in this investigation was 2.5%. A prevalence rate of 12.8% was recorded for Kaduna state with Kaduna South local government area recording the highest rate of 29.4% while Jema'a had the least of 6%. The high rate observed in Southern Kaduna compared to that of Plateau could be due to the fact that the Southern part of Kaduna State serves as a major trade centre for pig dealers coming from the south-western part of the country to buy pigs. Since the virus is very resistant to inactivation in the environment (Scott, 1965) and because of its great stability in blood and excretions (Plowright *et al*, 1968), it was possible that the high rate observed could be due to movements of contaminated waste equipment (e.g. ropes and other utensil used by the pig dealers in restraining the pigs), vehicles, and even contamination of wears, shoes etc. worn by the dealers themselves, from south-western Nigeria. Since ASF was reported to have gained entry to Nigeria from Benin Republic through the south-western region of Nigeria (Anon, 1998), therefore, frequent movements of pig dealers from endemic south-west region to southern part of Kaduna state to buy pigs could explain for the high rate

observed in that area. The result might also suggest that ASF is on the increase in that state. It was of interest to notice the absence of ASFV antibodies in pigs from Kachia local government area. Failure to detect antibodies to ASFV from the serum samples from that local government area does not imply absence of this disease in the area, as one of the tissues tested for ASFV antigens from that area gave a positive result. Besides, infected pigs respond immunologically by producing several types of antibodies that are only readily detected 5-6 weeks after the onset of illness (Malmquist, 1963). Therefore, sampling was probably done before detectable antibodies were produced.

The successful detection of antibodies to ASFV from the two states suggests that the disease was present in these states.

In this present investigation, the sex specific rate for males (7.4%) was significantly different from that of the females (3.6%). This findings differs from previous authors (Wilkinson, 1981; Plowright, 1984) who had variously reported that there is no difference in the distribution of infection amongst pigs in terms of age or sex and that pigs of all ages and sexes are equally susceptible. This discrepancy could be explained due to variations in sampling techniques and perhaps differences in locations of the studies.

The most widely employed technique for the detection of antigens to ASFV in outbreaks is the direct immunofluorescence (DIF). Experience with this technique is essential because it is used to confirm negative or doubtful results by ELISA in many affected countries (Escribano *et al*, 1990). Those tissues that

tested negative for antigens to ASFV by DIF were retested for virus DNA by polymerase chain reaction (PCR). Results obtained by the PCR revealed that, out of a total of 9 tissues (4 spleen and 5 lymph nodes) that tested negative for ASFV viral antigens by DIF and 1 tissue (lymph node) that tested positive for ASFV antigens by DIF, all the sample were positive for viral DNA except 1 (spleen). Failure to detect antigens for ASFV from these hitherto negative tissues could be due to poor keeping quality during transfer to the laboratory or virus antigen was masked by early antibody produced during infection and hence rendered it undetectable during DIF. However, PCR technique was able to identify the virus DNA from those hitherto negative tissues. This result makes the use of PCR advantageous over DIF particularly for identifying virus DNA in pig tissues which are unsuitable for antigen detection because they have undergone putrefaction, or when there is good reason to believe that virus may have been inactivated before samples are received in the laboratory.

## 5.2 Conclusion and Recommendation

The present study revealed the prevalence of antibodies to ASF in pigs from parts of Plateau and Kaduna states using I-ELISA to be 2.5% and 12.8% respectively. Antibodies to ASF were detected in all the local government areas considered from southern Part of Kaduna state except Kachia. The prevalence of ASF determined by the serological survey was found to vary from each local government.

The detection of antibodies and antigens to ASFV from the two states suggest that the diseases is present in these states.

Information obtained in this study could be of immense importance in planning for the control of the disease since an effective vaccine does not exist. In view of the growing awareness of ASF as a front-line disease of major economic importance to the pig industries of both the developed and developing countries, it becomes imperative that a nationaliy co-ordinated control programme against the disease is instituted. In doing so, there is the need for a considerable co-operation between the pig farmers and government veterinary authorities.

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## APPENDIX I

Biological reagents used were supplied by INIA (Instituto Nacional de Investigaciones Agrarias) Madrid, Spain. They include:

1. ASFV Positive antigen (Batch A204)
2. ASFV Negative serum (Batch A23)
3. ASFV Positive Serum (Batch A69)
4. Protein - A- Horse radish peroxidase conjugate
5. ASFV antibody conjugated to fluorescein
6. ASFV Positive Control tissues
7. ASFV Negative control tissues.

Indirect Enzyme Linked Immunosorbent (I-ELISA)

a. Reagents and Buffers used for the I-ELISA were prepared as follows:

i. Coating buffer for ASF antigen (0.05M Carbonate/bicarbonate buffer) was prepared as follows:
$NaHCO_3$ 4.2g
$Na_2CO_3$ (anhydrous) 5.3g
Distilled $H_20$ 1.0 litre
This buffer was maintained at pH 9.6 and stored at +4°C until ready for use.

ii. Wash buffer (Saline/Tween 20) was prepared as follows:
NaCl 8.5g
Tween 20 0.5ml
Distilled $H_20$ 1.0 litre
This buffer was maintained at pH 7.2 and stored at +4°C until ready for use

iii. Diluent for Sera and Conjugate (PBS/Tween 20) was prepared as follows:
NaCl 8.0g
KCl 0.2g
$Na_2H\ PO_4.12H_2O$ 2.9g
Tween 20 0.5ml
Distilled $H_2O$ 1 litre
The pH was adjusted to 7.2 and stored at +4°C until ready for use.

iv. Preparation of Chromogen - O.P.D. (Ortho-Phenylenediamine).
One tablet OPD was dissolved in 75ml distilled water. This preparation was allowed to completely dissolve and was stored in 25ml aliquots and stored at +4°C until ready for use.

v. Preparation of Substrate: $H_2O_2$ (3%) as follows:
$H_2O_2$ 1 tablet
Distilled water 10 ml

The substrate was stored at +4°C in the dark until ready for use.

vi. Preparation of Stopping Solution (1 M $H_2So_4$)
Concentrated $H_2So_4$ 16.1 ml
Distilled $H_2O$ 200 ml
The solution was left at room temperature until ready for use.

vii. Disinfectant Solution was prepared as follows:
VIRKON Powder 25.0g
Tap $H_2O$ 5 litres

# APPENDIX II

Date ..... Plate No ..... Operator .....
Antigen ..... Diluent .....
Neg. Serum ..... Pos. serum .....
Test sera ..... Sera Diluent .....
Conjugate ..... Diluent .....
Chromogen-Substrate ..... Diluent .....
Stopping sol n ..... Washing sol n .....

Incubation - Antigen, antibody and conjugate states at 37oC for 1 hour on shaker. Antisera may be incubated at 4oC overnight. Chromogen-substrate at room temperature for approximately 10 minutes. All diluted 1 in 50

(+) - ASF negative pig serum (+) - ASF positive pig serum (+) - Test sera

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | - | 1 | 2 | 3 | 4 | 5 | | | | | | + |
| B | - | | | | | | + | | | | | |
| C | | | | | | | + | | | | | + |
| D | | | | | | | - | | | + | | |
| E | | | | | | | - | | | + | | |
| F | | | | | | | | | | | | + |
| G | + | | | | | | | | | | | - |
| H | + | | | | | | | | | | | - |

Mean O.D. Values of Duplicate

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |

A positive result is indicated if the mean O.D. of duplicate wells is greater than twice the mean O.D. of the negative serum

## APPENDIX III

### Direct Immunoflourescence: PROTOCOL

i. P.B.S. solution: was prepared as follows:

NaCl 8.0g
$KH_2PO_4$ 0.2g
$Na_2HPO_4 .12H_2O$ 2.9g
KCl 0.2g
Distilled $H_2O$ 1000 ml.

This buffer was maintained at $P^H$ 7.2 and stored at +4°C until ready for use.

ii PBS – glycerol (10%) was prepared as follows:-
Glycerol ..... 1.0ml
PBS ..... 9.0ml.

iii Forty (40) microscope slides were placed into 100% ethyl alcohol for at least 1 hour before commencement of work so as to degrease the slides.

Iv Disinfectant solution was prepared as follows:-

I Virkon® powder ..... 25.0g.

Ii Tap Water ..... 5 litres.

## APPENDIX IV

### DNA Exraction and PCR - PROTOCOL

1. **L6** lysis buffer: 60g of GUSCN was dissolved in 50ml of 0.1ml tris HCL ($P^H$6.4). 11ml of 0.2M EDTA solution and 1.3g of Triton X – 100 (4°C) were added and allowed to completely dissolved at 50°C then stored at 4°C until ready for use.

2. **L2** wash buffer: 60g of GUSCN was dissolved in 50ml Tris HCL ($P^H$6.4) by placing at 50°C.

3. Preparation of size fractionated silica.6g of silica dioxide (Sigma) was suspended in a 50ml volume of dd $H_20$ in a 50ml glass cylinder and was allowed to sediment for 24 hours at room temperature.43ml of the supernatant was removed by suction and distilled water was added to a final volume of 50ml in the cylinder. The silica pellet was resuspended by vigorous shaking. Another second sedimentation was done for a period of 5 hours at room temperature. Then 44ml of the supernatant was removed and 60ul of HCL (32%w/v) was added in order to adjust the $P^H$ to 2.0. They were then dispensed into sterile bottle at 400ul per bottle and stored in the dark until ready for use.

4. Loading buffer:- 0.25% of Orange G in aqueous solution of 30% glycerol.

5. TAE buffer (50X) for agarose gel.
   Tris base (242g) glacial acetic acid.
   (57.1ml), 0.5M EDTA $P^H$ 8.0.

6 Marker DNA: Commercially available. 100bp ladder

7. Ethidium bromide (10mg/ml): i.e. 1g in 100ml of water. It was stirred using magnetic stirrer for several hours in order to dissolve the dye. The solution was then transferred into a dark bottle and stored at room temperature.

**Appendix V: ASF outbreak in Nigeria - State Reports**

*Source: NVRI Vom*

AFRICAN SWINE FEVER (ASF) OUTBREAK IN NIGERIA - STATE REPORTS*

| STATE | AFFECTED LGAs | LABORATORY REPORTS | TOTAL NO DEAD | ESTIMATED LOSS (N) |
|---|---|---|---|---|
| OSUN | Ife South, Iwo ,Ayedire,Ola-Oluwa, Ilesha East, Ilesha West, Oriade, Olorunda Ifelodun | Confirmation from Iwo sample | 1167 dead | N5835,000.00 |
| LAGOS | 10 LGA severely affected Including Badagry,Ojo, Agege, Epe, Ifako/Ijaiye, Mainland, Etiosa, Alimosho | No Laboratory confirmation | 45,58o dead on affected farms so far | 227,930,000.00 |
| OGUN | 4 LGAs atiected Ipokia, Yewa North, Yewa South,Imeko/Afon | No Laboratory Confirmation | 11,083 dead out of 11,415 on affected farms | 55,415,000.00 |
| DELTA | Gboloda, UbaleKekere &Beku in the estuary of Benin River,Ozoro, Warri, | Laboratory confirmation from Ozoro. Warri sample awaiting testing | No figure available (NFA) | - |
| RIVERS | One case from Port-Harcourt Commercial Piggery | Sample submitted[awaiting testing] | No figure available | Loss estimated at over N1.5m |
| KADUNA | Zonkwa Zongo Katafa,Kafanchan Jemmda, Kaduna (3) | Confirmed case from Zonkwa | 60,000+ - as reported in Guardian | 300,000,000.00 |
| BENUE | State wide except for 2 LGAs of Logo, Ukum. | Confirmed Gboko,Makurdi samples | 81900 + so far | 409,500,000.00 |
| AKWA-IBOM | Ikot- Ekpene LGA & Uyo LGA | Sample submitted[awaiting testing] | NFA | - |
| ENUGU | Udenu LGA. Nsukka LGA,Enugu, Enugu East | Sample awaiting testing | 321 dead out of 1212 | 1,605,000.00 |
| ONDO | Ilaje, Ese-Odo | No sample submitted | 745 dead out of 764 | 3,725,000.00 |
| OYO, EKITI, KOGI, EDO & KWARA. | NO REPORTED OUTBREAK TO DATE | | | |

**** REPORT AS AT DECEMBER, 31ST 1998***